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Divergent Evolution of Hepatitis C Virus in Liver and Peripheral Blood Mononuclear Cells of Infected Patients

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In infected individuals, hepatitis C virus (HCV) exists as a variably complex population of related genetic variants known as quasispecies. The quasispecies of HCV were studied previously in 10 chronically infected patients by single-strand conformation polymorphism analysis of a segment of the envelope gene E2/NS1 containing the hypervariable region 1 and it was found that certain variants (LC variants) were present both in the liver and in peripheral blood mononuclear cells (PBMC), others (L variants) were present in the liver but not in the PBMC, and still others (C variants) showed the opposite distribution. The sequence data obtained from nine such patients are reported, indicating that, within individual subjects, L and C variants are distinct phylogenetically. Results are described on the growth of HCV in stimulated healthy donor PBMC cultures supporting the concept that genetic divergence might stem, at least in part, from virus adaptation to growth in different cell types. This information may help to understand how HCV persists and produces disease in infected patients, especially with regard to extrahepatic pathology. ***J. Med. Virol. 57:57–63, 1999.***

KEY WORDS: hepatitis C virus; quasispecies; tissue compartmentalization

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic liver damage throughout the world and has also been associated with extrahepatic diseases, including mixed essential cryoglobulinemia, porphyria cutanea tarda, and membrano-proliferative glomerulonephritis [Gumber and Chopra, 1995]. An increased incidence of lymphatic neoplasia has also been noted in HCV-infected patients [Silvestri et al., 1996].

The positive-sense RNA genome of HCV is fairly well characterized, but natural history of the virus and mechanisms of persistence and pathogenesis are poorly understood. Similar to other RNA viruses [Domingo and Holland, 1997], HCV exists in infected hosts as a variably complex population of related genetic variants known as quasispecies [Martell et al., 1992; Wilson et al., 1995]. In a recent report [Maggi et al., 1997], the quasispecies of HCV were studied in chronically infected patients by single-strand conformation polymorphism (SSCP) analysis of a segment of the envelope gene E2/NS1 containing the hypervariable region 1 (HVR1) and it was found that genetic variants could be sorted into different groups. Certain variants (LC variants) were detected both in the liver and in the peripheral blood mononuclear cells (PBMC), others (L variants) were present in the liver but not in the PBMC, and still others (C variants) showed the opposite distribution. In addition, while most but not all of the variants detected in plasma could be categorized as LC, L, or C, a few (P variants) were found solely in plasma.

The sequence data show that HCV may undergo a divergent evolution in the liver and PBMC of infected patients and in vitro results support the concept that this divergence might stem, at least in part, from virus adaptation to growth in different cell types.

MATERIALS AND METHODS

Patients and Samples

The patients included in the study were 9 of the 10 patients studied in a previous report (one patient's specimens were no longer available) [Maggi et al., 1997]. Eight were infected with HCV genotype 1 and one (patient A6) with HCV genotype 2. Liver biopsy diagnosis ranged from persistent chronic hepatitis (patients A37, A46, and A55) to active chronic hepatitis

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(patients A6, A19, A24, A28, and A78) and cirrhosis (patient B1). None of the patients had received antiviral treatment at the time of the study. Serum HCV RNA titers ranged from <0.2 to 6.1 MEq/ml by bDNA 2.0 assay (Quantiplex HCV 2.0, Chiron, Emeryville, CA). Liver, PBMC, and plasma samples were obtained on the same day and processed as described [Maggi et al., 1997].

PCR Amplification and Analysis of HCV Quasispecies

Viral RNA was first reverse-transcribed and then amplified with primers covering the E2/NS1 region to obtain a 351-bp fragment spanning from nucleotide (nt) 1284 to 1634 of HCV genome as previously described [Maggi et al., 1997]. The PCR product was separated on 1.5% agarose gel, then extracted from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. Asymmetric PCR was carried out using the purified product with the same conditions as for the second-round PCR, except for the use of only the inner-sense oligonucleotide as primer. SSCP analysis was performed exactly as described [Maggi et al., 1997]. Briefly, a volume of single-strand DNA products was mixed with two volumes of formamide loading dye (95% deionized formamide, 20-mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) and electrophoresed in 10% nondenaturing polyacrylamide gel containing 0.5 × TBE buffer, 1.5% N,N′-methylenebisacrylamide, and 5% glycerol. Electrophoresis was carried out on the Dcode Universal Mutation Detection System apparatus (Biorad, Hercules, CA), immediately after loading, at 23°C with 40 mA for 4–5 hr; finally, the gel was visualized using silver staining.

Electrophoretic DNA mobility was read using a GDS 7500 Gel Documentation System (UVP, Cambridge, U.K.) and SSCP bands were analyzed by GelBase Pro software program (UVP). As previously described [Maggi et al., 1997], in preliminary SSCP experiments, different technical parameters were systematically studied to test the reproducibility of results. The amount of gel-loaded ssDNA, the gel composition, and the electrophoretic temperature were extensively controlled and then optimized. Multiple RNA extractions and PCR amplifications followed by independent SSCP analysis were undertaken on selected samples for which the amount of extracted RNA was sufficient to repeat the experiments. With the optimized conditions used, it was possible to discriminate by SSCP viral sequences differing at a single nucleotide position and, in accord with other reports [Enomoto et al., 1994; Nagasaka et al., 1996], to detect sequences representing approximately 5% of the entire population of variants. Previous studies had shown that SSCP can detect single-base polymorphism in PCR products with sensitivities of 80% or more, depending on DNA fragment length [Beier et al., 1993; Vidal-Puig and Moller, 1994].

Sequencing and Analysis of Viral Variants

Following silver staining, the SSCP gels were washed in distilled water for 1 hr. The bands were then excised from the gels and dissected into pieces of approximately 2 mm^2 in size, which were directly added to PCR mix and amplified. Sequence reactions of PCR products were undertaken with an automatic DNA sequencer (Pharmacia, Uppsala, Sweden). The DNA sequences were aligned and manually adjusted as described [Pistello et al., 1997]. Pairwise genetic distances were determined by Kimura's two-parameter model [Kimura, 1980], and the percent nucleotide sequence similarities calculated by subtracting the distance values to one and multiplying by 100. Phylogenetic analysis was carried out both by the Fitch-Margoliash method with pairwise distance matrix and 100 bootstrap resampling of the sequences, and by using the maximum-likelihood method with randomized data input [Felsestein, 1993]. Synonymous and nonsynonymous nucleotide p distances were calculated with the MEGA program [Kumar et al., 1993]. Amino acid sequences were established with the PROTEIN program (developed and available at Italian EMBnet Node, National Research Council, Bari, Italy), which assigned the 20 amino acids to 8, 4, and 2 different groups based on the chemical, functional, and hydrophobic properties of each amino acid. The output sequences were aligned and similarities were examined by plotting multiple alignments using PLOTSIMILARITY (Genetics Computer Group, Madison, WI). Sequence data and genetic distances are available by e-mail. Numbers of charged amino acids and net charge of the protein sequences spanning the HVR1 domain were calculated manually and by using PC-GENE software package (IntelliGenetics, Geel, Belgium), respectively. Statistical evaluation of the data was carried out by median nonparametric test and Wilcoxon rank sum test.

Infection of PBMC Cultures

PBMC were pooled from three HCV-negative individuals and stimulated with phytohemagglutinin (Sigma, St. Louis, MI) for 2 days. Aliquots of 2×10^6 of phytohemagglutinin-stimulated PBMC were then incubated at 37°C for 4 hr with 0.3–0.5 ml of infected plasma, containing between 0.6×10^5 and 1.8×10^6 HCV genome equivalents as determined by the bDNA 2.0 method (Chiron). After removing the inocula, the cells were washed twice with sterile phosphate-buffered saline and then transferred into flasks containing 5 ml of RPMI 1640 (Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum (Hyclone, Oud-Beijerland, Holland), and 20 U/ml recombinant human interleukin-2 (Boehringer Mannheim, Germany) and weekly monitored for the detection of positive- and negative-strand HCV RNA by diagnostic [Vatteroni et al., 1994] and tagged RT-PCR [Cribier et al., 1995] in the 5′ untranslated region, respectively. Finally, RNA extracted from the cultures found HCV-positive at 15

TABLE I. Genetic Variants of HCV Sequenced From Nine Chronically Infected Patients

Type of variant	Number of variants/patient[a]								
	A6	A19	A24	A28	A37	A46	A55	A78	B1
LC	1	3	2	2	2	2	2	4 (5)[a]	3
L	2	2	3	2 (4)	3 (4)	4		(1)	1
C		4	1	2	6 (7)	5	3	3 (4)	2
P	1		1 (3)	1	1	1		1	

[a]In parenthesis, the numbers of variants detected by SSCP analysis when different from the number of variants sequenced.

days postinfection was used for quasispecies examination by SSCP and sequencing.

RESULTS

Analysis of HCV Variants From Infected Patients

Collectively, SSCP analysis of HCV found in the liver, PBMC, and plasma samples from the nine patients included in the present study identified a total of 79 genetic variants of the virus. Since nine variants failed to generate sufficient amounts of template, only 70 could be sequenced and analyzed: 17 were L variants, 26 C variants, 21 LC variants, and 6 P variants (Table I). No sequence had stop codons or length polymorphism. In the 66 sequences from the eight genotype 1-infected patients (total 19,998 nt), position changes relative to the consensus sequence were 24% in the HVR1 domain and 5% in the flanking regions.

The intrapatient and interpatient percent nucleotide sequence similarities existing between the major groups of variants are shown in Figure 1. In each patient, similarity was lower when the C sequences were compared to the L sequences (median for the nine patients 82%) than when the C sequences were compared to the other C sequences (91%) or the L sequences to the other L sequences (99%). The LC variants showed an intermediate degree of divergence both among themselves and when compared to the C or L variants (data not shown). The degree of divergence exhibited by intrapatient C and L variants was similar to what was observed in interpatient comparisons (Fig. 1).

For each of the six patients with sufficient quasispecies complexity to make such analysis meaningful, a phylogenetic reconstruction of genetic lineages of the sequences was also carried out. Because analysis of viral quasispecies can be affected by artifacts due to mistakes in nucleotide incorporation occurring during reverse transcription and PCR amplification [Smith et al., 1997], to minimize the problem, the trees were obtained after removing from the sequences the nucleotide substitutions found in only one of the patient's sequences, with the exception of those substitutions representing the only distinctive trait of variants present in more than one tissue. It was reasoned that nucleotide changes present in more than one sequence were likely to represent genuine polymorphism actually present in the original virus population. As shown by Figure 2, in each case the C sequences showed a clear tendency to segregate together and separately from the L sequences and the segregations were supported by high bootstrap values, while LC and P sequences were intermingled. These patterns were confirmed by submitting to similar analysis the entire unprocessed sequences and the HVR1 segment only as well as by using the maximum-likelihood method (data not shown).

The deduced amino acid sequences were then examined for distinguishing characteristics. No signature amino acids or patterns distinctive of the L or C variants were detected manually or by computer-assisted examination of chemical, functional, and hydrophobic features of the protein sequences. However, on average the HVR1 domains of the C variants had a significantly higher charge at pH 7 (Fig. 3) and contained significantly larger numbers of charged amino acids (Table II) than the L variants. Of the potential glycosylation sites, two located in the 5′ flank of HVR1 were highly conserved but one, located 3′ of HVR1, was present in approximately 1/4 of the C and LC variants and never found in the L variants (data not shown). Collectively, these findings were compatible with the concept that HCV quasispecies can evolve independently in the liver and PBMC, possibly due to differences in viral surface charge and/or conformation of the viral envelope proteins capable of influencing cell-type preference.

HCV Quasispecies Changes in PBMC Cultures

To investigate whether the divergence between liver and PBMC quasispecies observed in infected patients was driven by the different cell types used for virus replication, the changes in HCV quasispecies resulting from HCV growth in cultures of stimulated PBMC were examined. Phytohemagglutinin-stimulated healthy donor PBMC were exposed to six of the plasma samples used in the first part of the study under conditions known to permit limited growth of the HCV [Cribier et al., 1995; Mizutani et al., 1996; Nakajima et al., 1996] and kept in culture for 15 days. At the end of the incubation period, four cultures were found virus-positive by diagnostic PCR. In addition, two of the cultures, exposed to 0.6×10^5 and 1.8×10^6 genome equivalents, respectively, were also positive for intracellular negative strand HCV at day 7, thus providing evidence of active virus replication. Day 15 positive cultures that had been exposed to plasma from patients A19, A28, and A37 were examined for quasispecies composition exactly as above. Similar to observations with other viruses [Spira and Ho, 1995; Strizki et al., 1996], growth in tissue culture led to a variably pronounced reduction of HCV quasispecies diversity, since the viral variants detected decreased from 9 to 7 (plasma A19), from 7 to 3 (plasma A28), and from 12 to 1 (plasma A37). More interestingly, analysis of the in vitro grown variants showed that their nucleotide sequences were identical either to C or to LC variants present in the original plasma inoculum (4 and 2 in the A19 and A28 cultures, respectively) or differed from input variants but, when submitted to phylogenetic

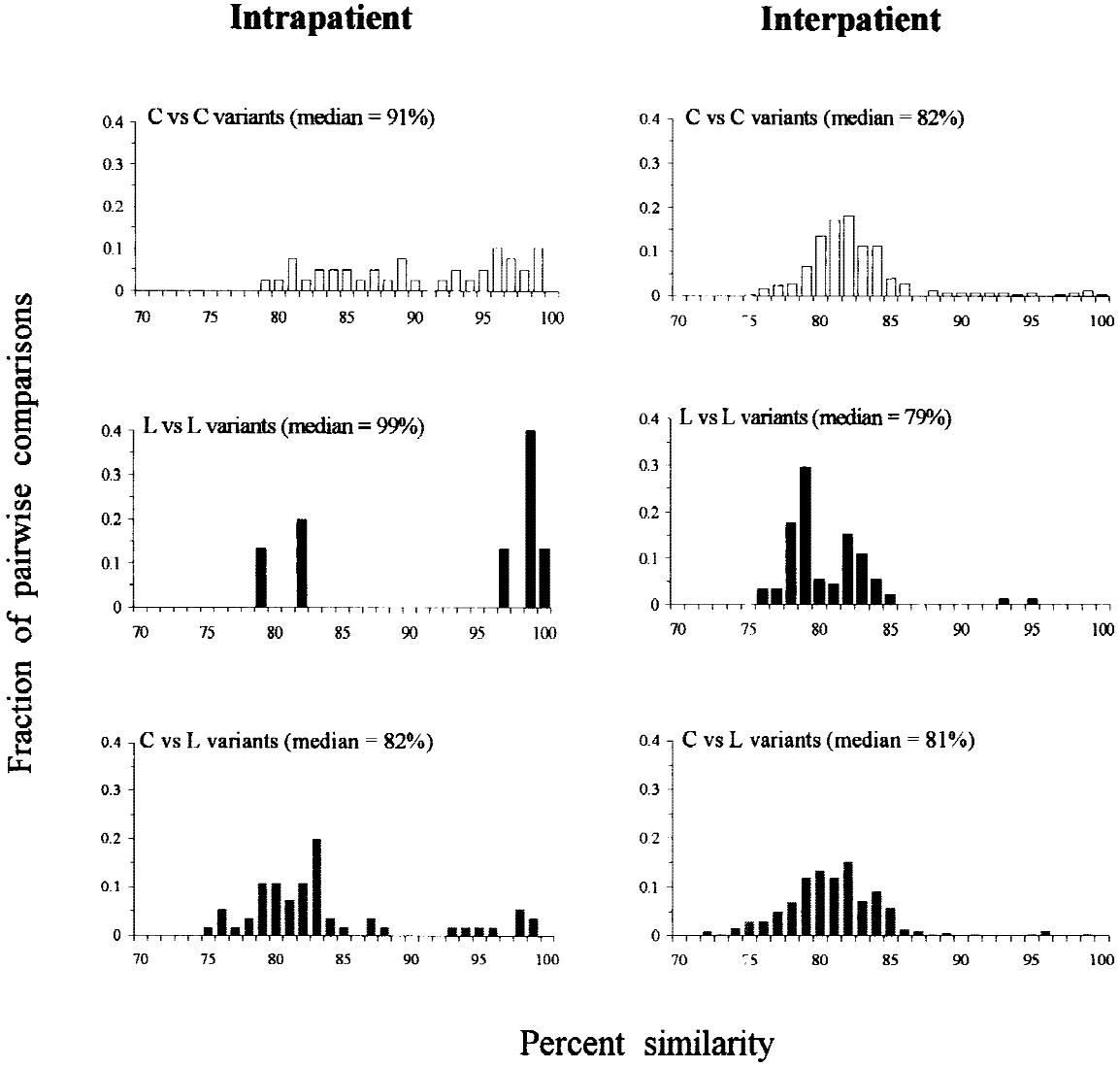

Fig. 1. Intra- and interpatient nucleotide sequence similarities distributions between HCV variants in a 303-nucleotide segment obtained by subtracting the primers nucleotide from the 351-bp region sequenced. The numbers were calculated and rounded off to the nearest percentage. Intrapatient analysis was performed for each of the nine study patients, whereas interpatient analysis only included the eight patients infected with genotype 1. Bars represent the fraction of the total number of pairwise comparisons showing the percent similarity indicated. Similarity was significantly greater within the C variants than between C and L variants both in the intrapatient (Wilcoxon rank sum test; $P < 0.001$) and in the interpatient analysis ($P < 0.001$).

analysis, showed a clear tendency to cluster with the C variants of the plasma donor (Fig. 2), while the L variants, initially well represented in the infecting plasma samples and the P variants, had become undetectable. These findings clearly indicated that growing HCV in cultured PBMC under the conditions used selected for variants typically associated with PBMC in vivo and that this selection was strong enough so that the other variants disappeared in a relatively short time.

DISCUSSION

HCV can exhibit different quasispecies composition in the liver, PBMC, and plasma of infected patients [Maggi et al., 1997], a conclusion that has been born out also by findings in other laboratories [Fujii et al., 1996; Gretch et al., 1996; Saito et al., 1996; Cabot et al., 1997; Shimizu et al., 1997]. It was also reported that, within individual patients, the genetic variants of HCV could be classified into different groups: L variants were present in the liver but not in the PBMC, C variants exhibited the opposite distribution, and LC variants were present in both tissues. Moreover, of the variants circulating in the plasma, most were identical to variants contained in the liver and/or PBMC, but a few were not, thus suggesting that they might derive from additional sites of virus replication [Maggi et al., 1997].

Further HCV quasispecies were studied by sequencing and analyzing 70 of 79 HCV variants obtained from nine of the patients included in a previous study. The

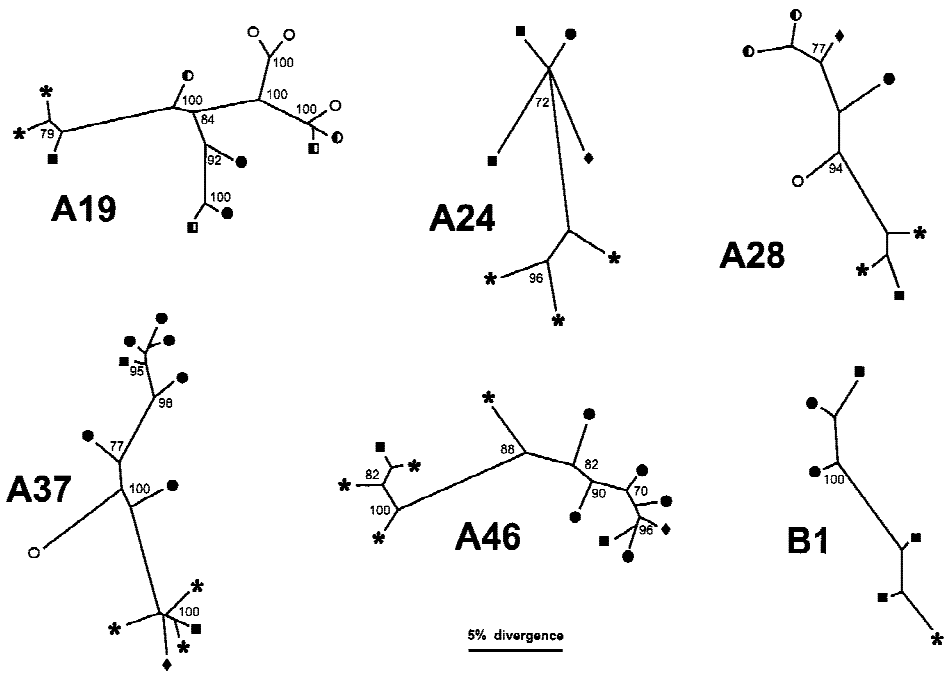

Fig. 2. Phylogenetic analysis of viral sequences from the six patients infected with genotype 1 HCV. The trees are supported by the bootstraps values indicated. Symbols represent: (●), C variants; (✱), L variants; (■), LC variants; (♦), P variants; (◐) and (◨), C and LC variants, which were maintained after cultivation in vitro; and (○), in vitro emerged variants, which were not present in the original plasma inoculum.

results show that, within each patient, similarity was significantly lower when the C sequences were compared to the L sequences than when the C sequences were compared to the other C sequences or the L sequences to the other L sequences, while the LC variants showed an intermediate behavior. Moreover, phylogenetic analysis showed that in each of the six patients with sufficiently complex quasispecies, the C sequences showed a clear tendency to cluster together and separately from the L sequences, while LC and P sequences were intermingled. Also, when the deduced amino acid sequences were examined for distinguishing characteristics, on average the HVR1 domains of the C variants had a significantly higher charge at physiological pH and contained significantly larger numbers of charged amino acids than the L variants, and potential glycosylation sites were more numerous in the C and LC variants than in the L variants. Taken together, these findings represent clear evidence that the differences in HCV quasispecies composition can stem at least in part from divergent virus evolution, even though variants with intermediate characteristics also exist.

That viruses may follow partially distinct parallel evolutionary pathways in different host tissues and anatomical sites has been shown for a number of viruses [Sankale et al., 1995; Van der Hoek et al., 1996; Domingo and Holland, 1997; Wong et al., 1997]. A recent report has shown that the same can occur in HCV-infected patients [Navas et al., 1998]. There are several possible reasons why partially divergent HCV quasispecies may form in different tissues, including spatial separation, cell-type–specific differences in viral binding, postbinding events or intracellular replication rates, and diversity in local immune-selective forces. The results described above point to cell tropism differences as an important fitness selection mechanism in the generation of tissue-specific HCV diversity. First, L and C variants had somewhat different envelope proteins, as determined by examining net charge and numbers of charged amino acids and potential glycosylation sites. Secondly, the ratio of synonymous to nonsynonymous site mutations were similar in the two types of variants (data not shown), thus suggesting that diversification was not due to differences in the immune pressure encountered by the virus in different tissues [Gojobori et al., 1990; Li, 1993]. Third and possibly more significant, short-term propagation in cultured stimulated PBMC restricted the viral quasispecies and selected for genetic variants associated with PBMC in vivo, thus showing that these variants have an evident replicative advantage in these cells.

On the other hand, this interpretation is in accord with recent evidence, obtained both by examining infected liver transplant recipients [Gretch et al., 1996], chimpanzees [Shimizu et al., 1997], and virus grown in vitro [Ikeda et al., 1997], showing that lymphotropic variants of HCV do indeed exist. In this scenario, the LC variants would seem to represent dual-tropic variants. Selective pressures that generated distinct liver and PBMC viral quasispecies and working on a site of the viral envelope surface glycoproteins different from those studied cannot, however, be excluded. It is possible that further analysis of HCV quasispecies tar-

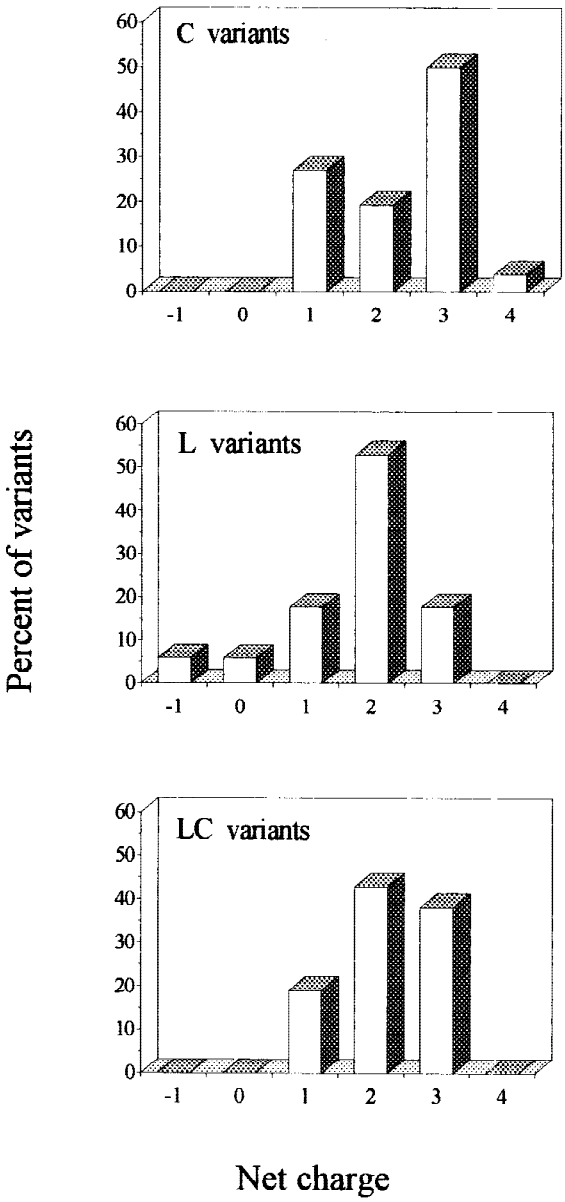

Fig. 3. Net charge at pH 7 of the deduced HVR1 domain. The median charge of C variants was significantly higher (2.94 ± 0.88) than that of L variants (1.99 ± 1.05; $P < 0.001$). LC variants had intermediate mean charge (2.0 ± 0.72).

TABLE II. Numbers of Charged Amino Acids in the HVR1 Domain of the 70 HCV Variants Sequenced

Type of variant	Number of variants with charged amino acids					
	1	2	3[a]	4	5	6
LC	0	2	10	4	5	0
L	0	3	5	7	1	1
C	1	4	16	3	2	0
P	0	1	3	2	0	0

[a]Significantly different for C versus L variants (median nonparametric test; $P < 0.05$)

geted to additional portions of the viral genome will permit recognition of determinants of cell tropism. The receptor binding region of HCV, as well as the corresponding cellular receptor, have yet to be identified.

In conclusion, these results encourage further studies of HCV quasispecies. They may help to better understand HCV biology, as well as the mechanisms behind HCV persistence and disease production, especially with regard to extrahepatic pathology [Gumber and Chopra, 1995; Hoofnagle, 1997].

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